OSMOTIC HATCHING IN THE EGGS OF SOME FRESH-WATER COPEPODS

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Hatching of the eggs of eucopepods apparently has been described only by Marshall and Orr (1954, 1955) and Ziegelmayer (1927). The two papers differ fundamentally in the interpretation that is given, for Ziegelmayer, working with 17 species of *Cyclops,* thought the outer egg membrane swelled over a period of 6 to 12 or more hours, developing a pressure between the inner and the outer membranes. Subsequently, according to him, the outer membrane would burst, and the nauplius emerge, closely surrounded by the inner membrane. On the other hand, Marshall and Orr thought that the inner membrane swelled, and that the pressure developing within it resulted in the rupture of the outer membrane. The inner membrane, containing the unhatched nauplius, emerged through the opening. These authors described a considerable space between the nauplius and the stretched inner membrane. Observations were made by Marshall and Orr (1954) primarily on the marine calanoid, *Calanus finmarchicus,* but they supplemented their study by the examination of other marine copepods belonging to four sub-orders (Calanoida, Cyclopoida, Harpacticoida, and Caligoida), and by the examination of two species of fresh-water cyclopoids (*Cyclops agilis* and *C. viridis*).[1]

From the general appearance of the hatching process, both Ziegelmayer and Marshall and Orr concluded that it was osmotically controlled. Although Ziegelmayer's paper was devoted largely to reports of experiments on the permeability and changes of permeability of the membrane, he performed no experiments that were aimed at proof of osmotic control. Marshall and Orr performed a rather inconclusive experiment, in which they placed 15 *Calanus finmarchicus* eggs that were nearly ready to hatch in sea water that had been diluted with a small quantity of fresh water. Eleven of the eggs bulged and 7 hatched successfully, whereas in the controls in undiluted sea-water only 3 out of 14 bulged and hatched.

In explanation of the onset of the hatching process, Ziegelmayer was convinced that there was a change of the permeability of the membrane, caused by some influence (hormone?) from within (hence, from the enclosed nauplius). On the other hand, Marshall and Orr (1954) suggested that it (p. 400) "might be that a sudden increase of excretion by the embryo leads to an increased content of salts and the imbibition of water."

Materials and Methods

Ovigerous specimens of *Diaptomus siciloides, D. ashlandi, D. oregonensis, Cyclops bicuspidatus* and *Mesocyclops edax* were taken from the plankton in

[1] After the present paper was in press, it was discovered that P. Heegaard in 1947 (Contribution to the phylogeny of the arthropods: Copepoda, in *Spolia Zool. Mus. Hauniensis,* 8: 1–227) had given figures and brief descriptions clearly indicating that hatching in *Caligus curtus, C. rapax,* and *Lernaeocera branchialis* occurs in a manner comparable to that described by Marshall and Orr (1954).

Hatchery Bay, Put-in-Bay, Ohio (western Lake Erie) in mid-June to mid-July, 1958 (air and water temperatures were 21° ± 1° C. during the period of collection and observation – 23° C. for *M. edax*). Most of the observations, and all of the experiments, were with *Diaptomus ashlandi* and *D. siciloides*. Specimens were kept in U. S. Bureau of Plant Industry model watch-glasses until chosen for detailed observations, at which time the egg sacs were removed from the mothers and the eggs observed with a compound microscope magnifying 100× and 443×. No coverslip was used, the objective being immersed directly into the water when necessary.

Observations of the hatching procedure were supplemented by experiments designed to test the validity of the osmotic theory of hatching. Sucrose solutions were made up of the following concentrations: 1 *M*, 0.5 *M*, 0.4 *M*, 0.3 *M*, 0.2 *M*, 0.1 *M*, 0.05 *M*, 0.04 *M*, 0.03 *M*, 0.02 *M*, and 0.01 *M*. These solutions were used to ascertain the approximate osmotic value of the fluid within the inner membrane, and to test the permeability to water of the inner membrane and larval surface at certain stages of the development of the nauplius in relation to the moment of hatching. To avoid repetition, the detailed experimental procedures are more conveniently given below under Results.

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Results

1. *Simple observations*

No difference was observed in the hatching of the eggs of *Diaptomus siciloides*, *D. oregonensis*, and *D. ashlandi*. Before hatching, the eggs averaged 109 μ in diameter, and thus had a volume of 678,110 μ^3. Individual eggs were separated from each other by the material of the egg sac proper, as shown in Figure 1, which depicts a portion of an egg mass of *D. siciloides*, with the eggs near the hatching stage. In this preparation the eggs were spread apart with fine needles for ease in viewing them, and one has been displaced from the egg sac. As shown, there are spaces of small but variable magnitude between the egg sac material and the eggs proper. Only a single membrane can be distinguished around the enclosed larva but in reality there are two, as shown below. The contained embryo for the most part fills the entire space within the membranes, although when viewed from the dorsal aspect, the naupliar appendages are visible laterally, closely appressed against the body. The red bi-crescentic naupliar eye is clearly visible anteriorly. The nauplius was observed to twitch its legs from time to time as much as 24 hours before hatching began.

The initiation of hatching was indicated by the appearance of a fluid-filled space between the nauplius and the egg membranes. This was followed very quickly by the bulging of the egg surface (Fig. 2). The outer membrane broke, probably due to the internal pressure, and it could be seen that there was a second inner membrane

protruding through the opening (Fig. 3). In eggs which were isolated from the egg mass, it was clear that the two halves of the outer membrane were pushed aside by the emerging inner membrane. For some time a portion of the inner membrane remained inside of one of the halves of the outer membrane (Fig. 4), but eventually the entire inner membrane slipped out as a perfect sphere, and left the outer membrane behind (Fig. 5). For all of this period the volume enclosed by the inner membrane was increasing, so that when the stretched membrane slipped free of the outer membrane it had an average diameter of 153 μ and a volume of 1,875,400 μ^3. Thus the volume, compared to the original volume of the egg, increased in a ratio of 2.77:1. The nauplius was completely surrounded by a fluid-filled space.

During the extrusion of the inner membrane from the outer, the unhatched nauplius typically remained completely motionless. In some instances it twitched, but never more than it had for several hours previously. At first after extrusion,

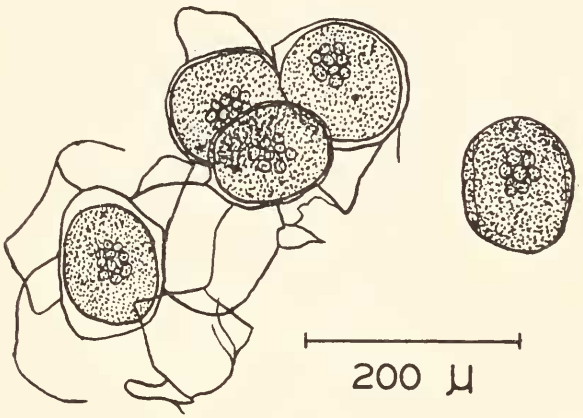
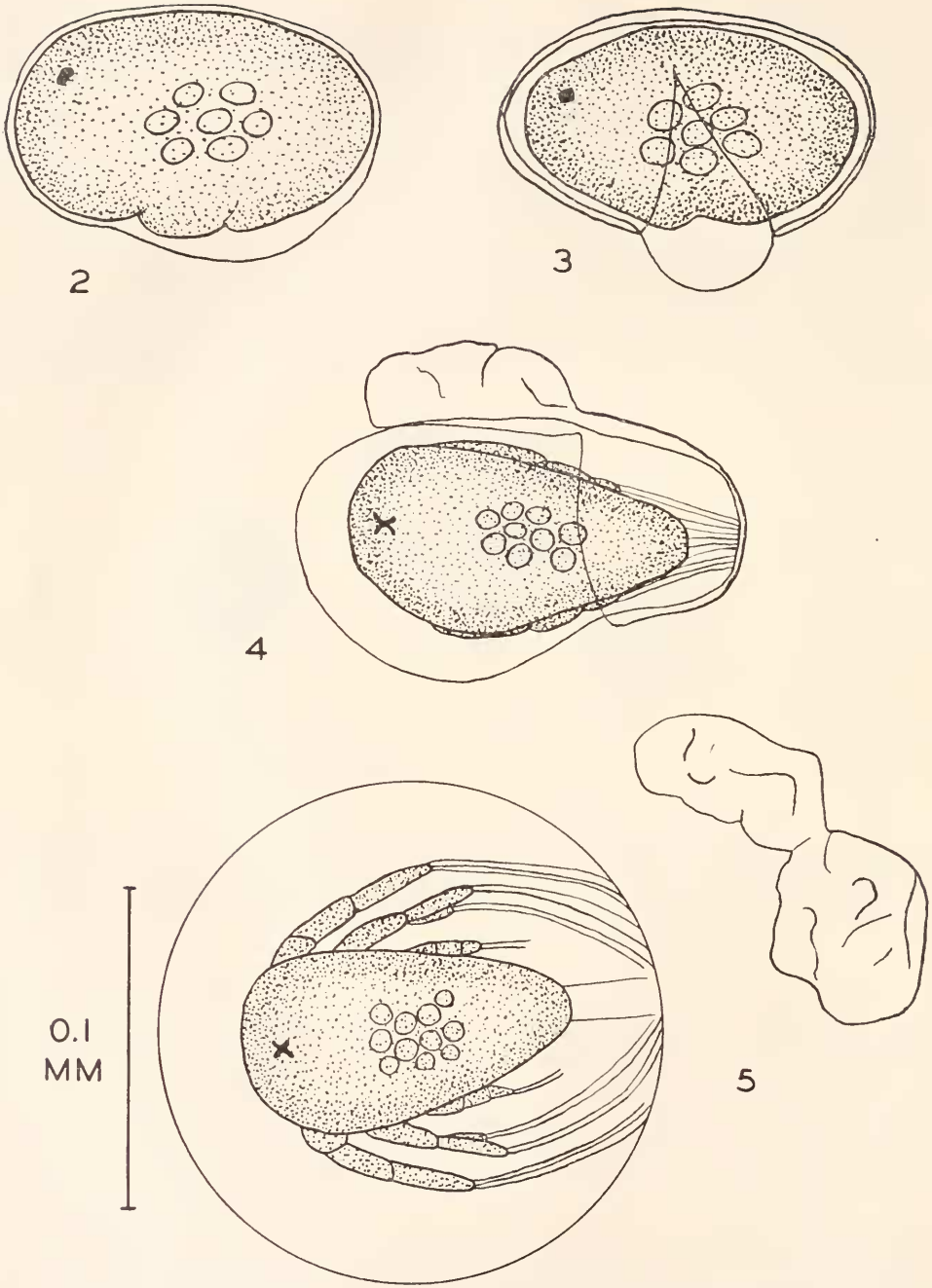


FIGURE 1. A portion of an egg sac of *Diaptomus siciloides* with eggs near the hatching stage. The sac was teased apart with fine needles, and one egg has been displaced from it. Note that the individual eggs lie somewhat loosely within the secretion forming the egg sac. Also note that the embryo is closely invested by the egg membranes, so that there is no space between the membranes and the enclosed nauplius.

the three pairs of appendages were held as they were before the swelling of the inner membrane. Although each larva that was observed at this stage was examined carefully, and larvae were seen from all possible angles, no trace of a third membrane, close around the animal, could be detected. After a period of one or two minutes, the appendages suddenly broke free from the sides of the body, and became extended laterally in their usual free-living naupliar position. This was observed many times. When the larva was viewed from a dorsal or ventral position, it could be seen that the appendages broke free from the sides of the body in a series of two or three short jerks. There was no evidence that this movement took place through muscular contractions of the animal (though this was not precluded). The *appearance* was that the appendages were, due to their structure and elasticity, pulling in the direction of their normal naupliar position, and that suddenly some tissue, membrane, or other material holding the appendages down tore loose from



FIGURES 2–5. Individual eggs of *Diaptomus siciloides* during hatching. Figure 2: a fluid-filled space has appeared between the egg membranes and the nauplius, and the membranes are bulging on one side. The inner and outer egg membranes are not yet distinguishable. Figure 3:

the strain. A few seconds after the appendages assumed their naupliar position, the animal began to move them in the twitching manner characteristic of free-swimming calanoid nauplii. Approximately a minute later the diaphanous membrane of the sphere burst with great suddenness. The internal fluid, being under considerable pressure, was forced almost explosively out through the breach, carrying the nauplius with it. While the nauplius was within the sphere, the setae of the appendages, and the setae at the posterior end of the animal, appeared as though they could easily and readily perforate the delicate membrane, for they impinged upon its surface as the animal continued its movements. However, this method of escape apparently did not occur during normal hatching, for almost invariably the larva escaped head first, whereas the setae touched the membrane at the opposite

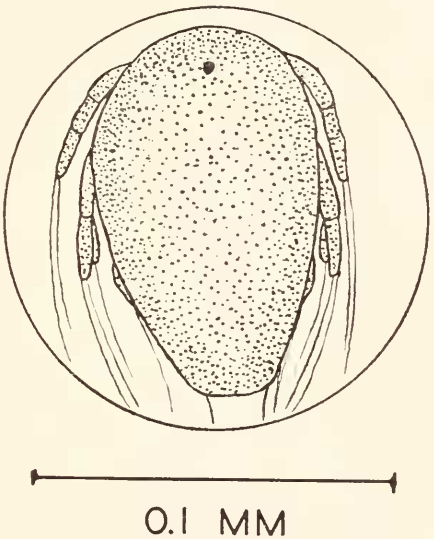


FIGURE 6. The nauplius of *Cyclops bicuspidatus* within the sphere formed by the inner egg membrane after extrusion.

side of the sphere. Upon close observation it could be seen that the end of each of the setae was pliable and bent over when it touched the membrane. Penetration by the sharp ends of the setae as suggested by superficial examination, could not occur (see below in section 4 for further observations on the breaking of the membrane).

The total time elapsing from the first indication of hatching to its completion ordinarily varied from 7 to 8 minutes.

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the outer egg membrane has burst, and the inner membrane is bulging out through the opening. The outer and inner membranes are clearly seen. Figure 4: the outer membrane covering the anterior half of the emerging larva has slipped off, but the inner membrane remains within the other half of the outer membrane. The naupliar appendages are closely appressed against the sides of the body. Figure 5: the inner membrane, expanded to its maximum diameter, has slipped out of the outer membrane entirely, forming a perfect sphere. The naupliar appendages have assumed their swimming position.

Hatching was observed in single sets of eggs from *Cyclops bicuspidatus* and *Mesocyclops edax*. It occurred in much the same manner as in the 3 species of *Diaptomus,* and took an average of 6½ minutes from start to finish. As with *Diaptomus,* the nauplius was passive during the swelling and protrusion of the inner membrane from the outer membrane and from the egg mass, except for a few twitches. The volume increase of the contents of the inner membrane was not quite as extensive as in *Diaptomus*. Before hatching the eggs averaged 82 μ in diameter. The spheres averaged 112 μ. Hence the average volume changed from 288,710 μ^3 to 735,655 μ^3, or a ratio of 1:2.55. The nauplius occupied a greater portion of the contents of the sphere than was the case with *Diaptomus* (Fig. 6), and it did not escape in the same explosive fashion. When the membrane surrounding the sphere burst, there was a sudden release of pressure, and the sphere collapsed, opening, as with *Diaptomus,* at the head end of the larva. In both of these cyclopoids, in all the instances observed, however, the nauplius remained somewhat entangled in the membrane, and escaped only after a short struggle.

2. *Experiments indicating the osmotic nature of hatching*

The general appearance of the hatching process in copepods strongly suggests that it is osmotically regulated. However, this has not been proven incontrovertibly by any experimental results heretofore reported.

In preliminary exploratory experiments, eggs of *Diaptomus siciloides* in the beginning stages of hatching were immersed in a 1 *M* solution of sucrose, or in double-distilled water. The permeability to water of the inner membrane and the naupliar surface was clearly shown by the fact that in the sucrose solution the larvae and the inner membranes shrank drastically from the outer membrane through the osmotic loss of water to the hypertonic solution. Obviously the larvae were destroyed (Fig. 7). In double-distilled water, on the other hand, hatching (*D. siciloides*) was completely normal except that the average time consumed during the hatching process was reduced to 6 minutes, compared to an average of 7½ minutes for the controls (the nauplii from the experimental eggs became turgid and weak in their movements after hatching, and died by bursting in 15 to 20 minutes).

A 0.1 *M* sucrose solution was used for another set of eggs. Some of the eggs already had hatched, one was in the process of hatching, and a group of five in the egg mass had not yet begun to hatch. The inner membrane of the hatching egg quickly shrank back against the larva. None of the larvae was obviously distorted from the osmotic effects of the solution, and they continued to twitch in a normal fashion. No sign of hatching was observed in any of the eggs. The eggs were maintained in the 0.1 *M* sucrose for 4 hours, at which time they were transferred to lake water. Immediately all of them began to swell. Spheres of normal size formed, but they were not entirely freed from the outer membranes. The nauplii were very weak. All of them hatched, but they died soon. It is thought that these deaths may have been the result of some other factor than osmotic effects, for example from anoxia. This is suggested by subsequent experiments and by the fact that some of the already hatched siblings of the experimental nauplii were placed in 0.1 *M* sucrose for 24 hours, then transferred to lake water, with no ill effects.

A set of eggs of *D. siciloides*, some of which were hatching, was placed in 0.05 *M*

sucrose solution. Some larvae began the hatching process. There was some swelling, but apparently insufficient pressure was built up to burst the outer membrane. The eggs were placed back in lake water after 30 minutes in the sugar solution. Hatching began immediately, the first larva being freed 8 minutes later.

Another set of eggs of the same species at hatching time was placed in 0.04 *M* sucrose solution. Those that had already formed spheres hatched. Those still in the outer membranes (including those that had started to hatch) failed to hatch or change in any way during 17 minutes. The eggs were then placed in 0.03 *M* sucrose. Swelling was immediate (in 3 out of 4 eggs). One of these hatched in about three minutes. Another swelled considerably but failed to squeeze out of the outer membrane. There was no further change for 15 minutes. The remaining

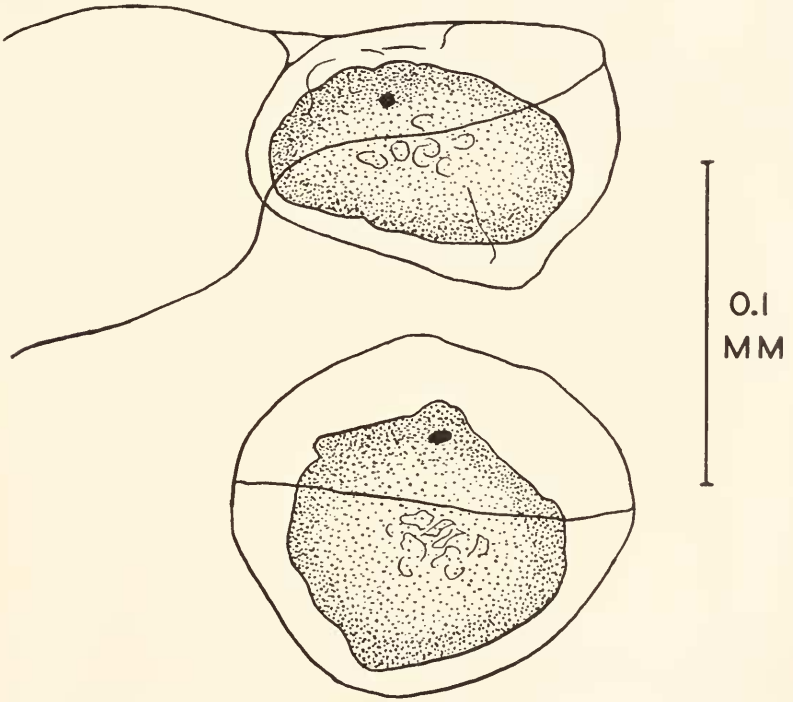

FIGURE 7. Two hatching eggs of *Diaptomus siciloides* after immersion in 1 *M* sucrose solution. The nauplii and the inner membranes have collapsed through the osmotic loss of water. The outer membranes are unshrunken, though somewhat contorted.

eggs were then placed in 0.02 *M* sucrose. In 2½ minutes one larva had hatched, but when the membrane broke, the larva was not thrown out. In another egg a sphere was formed (136 μ in diameter—considerably smaller than the average). When the membrane broke, it did so slowly, taking a full two seconds to collapse. The larva was not thrown out in the usual manner, but temporarily remained entangled in the collapsed membrane.

The results described above are summarized, along with additional information, in Table I.

The resistance of the outer membrane of eggs in the early stages of hatching interfered with efforts to ascertain the approximate osmotic pressure of the fluid within the expanded inner membrane. It was necessary to experiment with spheres that already had been extruded (thus very shortly before completion of hatching). Successful observations were completed in 18 instances, results being similar both for *D. ashlandi* and *D. siciloides*.

The results obtained with isolated extruded spheres are condensed in Table I. The following are representative experiments: 1) A nauplius of *D. ashlandi* in the twitching stage in a sphere was placed in 0.05 *M* sucrose. The diameter of the sphere decreased very gradually over a period of 12 seconds (plus an unknown portion of the duration of time needed to find it under the microscope). When all the space within the membrane had disappeared, the larva moved, puncturing the membrane with one of its antennae, after which it escaped. 2) A nauplius of *D. ashlandi* in a sphere was placed in 0.04 *M* sucrose. The sphere shrank. It was then placed back in lake water where it swelled up again. It was placed in 0.04 *M* sucrose a second time, and shrank again. The nauplius then moved, punctured the membrane, and escaped. 3) A larva of *D. ashlandi* in a sphere was placed in

TABLE I

Summary of the effect of various concentrations of sucrose on the swelling and hatching of eggs. (+ = occurring, ± = sometimes occurring, and sometimes not, − = not occurring.)

Sucrose conc. (*M*)	Swelling of intact egg	Bursting of outer membrane	Extrusion of inner membrane	Swelling of inner membrane	Hatching from extruded sphere	Shrinking of inner membrane
Lake water	+	+	+	+	+	−
0.01	+	+	±	+	+	−
0.02	+	±	±	+	+	−
0.03	+	±	±	±	±	±
0.04	−	−	−	−	±	+
0.05	−	−	−	−	±	+
0.10	−	−	−	−	−	+

0.03 *M* sucrose. The membrane shrank somewhat, but a considerable space remained between the nauplius and the membrane. The membrane no longer formed a perfect sphere, but was distorted to an ovoid shape with dimensions of 119 $\mu \times$ 146 μ. The larva hatched successfully.

From the results of such experiments, it appears that the osmotic pressure of the fluid within the extruded inner membrane was approximately equivalent to that of 0.03 *M* to 0.04 *M* sucrose. Such solutions have osmotic pressures of 0.672 to 0.896 atmosphere. The Δ of the fluid (Δ_f) would be 0.056 to 0.074. The freezing point of the Lake Erie water used was −0.03° C. The Δ of the internal fluids of nauplii has never been measured, but Δ_i for fresh-water Crustacea, as summarized by Krogh (1939) and Harnisch (1951), lies between 0.30 (*Daphnia magna*) and 0.81 (*Potamobius*). Przylecki (1921), reported by Krogh (1939), observed the Δ of older eggs (50–80 hours) of *Daphnia magna* to be 0.74. It would therefore appear that Δ_i is greater than Δ_f, which in turn is greater than Δ_o.

The above reported results indicate very clearly that the hatching of the copepod eggs studied here was by osmotic means.

3. *Test of the permeability of the egg membranes*

Ziegelmayer (1927) concluded that a change in the permeability of the (outer) membrane initiated the process of hatching. In contrast, Marshall and Orr (1954) suggested the possibility that there was a sudden increase of the osmotic pressure of the fluid within the inner membrane, after which hatching proceeded. To the present author this latter hypothesis seemed reasonable, for the alternate hypothesis apparently would be that the non-living inner membrane would have to change its permeability suddenly. Such a sudden change certainly would not be unexpected in a living membrane, but would not be as likely for a non-living membrane. Therefore the results reported below were unexpected.

As a preliminary experiment, to test the effect of some of the higher osmotic concentrations on the nauplius itself, larvae of *Diaptomus siciloides* which had just hatched were placed in a series of sucrose solutions as follows: 1 *M*, 0.5 *M*, 0.4 *M*, 0.3 *M*, 0.2 *M*, and 0.1 *M*, with other nauplii remaining in lake water as controls. Larvae became contorted and succumbed instantly in 1 *M* sucrose. In a 0.5 *M* solution they died within a few seconds, and likewise showed distinct evidence of the osmotic removal of water from their tissues. Results were the same in 0.4 *M* sucrose, though the larvae lived somewhat longer. In 0.3 *M* sucrose they lived over ten minutes, but the end result was similar. In the 0.2 *M* solution, at the end of 10 minutes they appeared normal, but moved seldom and weakly. Subsequently they died. In the 0.1 *M* solution they lived normally for many hours, but moved somewhat less vigorously than did the controls.

Thus the larvae can withstand a solution with an osmotic pressure as great as that of 0.1 *M* sucrose, but not as great as that of a 0.2 *M* solution.

In a subsequent experiment, an egg sac of *D. ashlandi* containing hatching eggs was placed consecutively in 0.1 *M*, 0.2 *M*, 0.3 *M*, 0.4 *M*, and 0.5 *M* sucrose, and observed for shrinkage of the inner membranes and of the enclosed nauplii. No shrinkage was apparent in the 0.1 *M* or 0.2 *M* solutions. A slight shrinkage could be seen in the 0.3 *M* solution, but it was somewhat obscure. In 0.4 *M* and 0.5 *M* solutions, however, shrinkage was considerable. The nauplii within the shrunken inner membranes appeared to be destroyed.

However, in three out of the ten eggs in the egg case, no shrinkage occurred, even in the 0.5 *M* solution, and the enclosed nauplii continued to twitch. A few minutes after being transferred to 0.5 *M* sucrose, one of the three suddenly began to shrink (not timed) but the other two remained as they were. Approximately one-half hour later the second one rather suddenly shrank. The third, on the other hand, still maintained life, and was intact, at the end of 2½ hours, although by this time (in the conditions of the experiment) considerable evaporation had occurred, and therefore the sugar concentration was higher than 0.5 *M*.

A second egg case of *D. siciloides* was placed in 0.5 *M* sucrose. Again, three out of ten eggs failed to shrink in the 0.5 *M* solution, but the remainder clearly showed the effects of the hypertonic external medium. Two of the three started to shrink 19 minutes after the egg case was placed in the sucrose, and the other began 2 minutes later. The process of shrinking took approximately 3 to 4 minutes (the time at which shrinking was completed was difficult to judge exactly). One-half hour after the egg case was placed in the sucrose, it was returned to lake water. Hatching was successful in nine of the ten eggs, though the nauplii were not normal (see below in section 4 for a more detailed description of this hatching).

These results suggest that in those eggs where hatching had begun, or was ready to begin, the inner membrane rather suddenly became permeable to water, whereas in those eggs not yet ready to hatch the inner membrane was impermeable.

To test the hypothesis that a permeability change takes place when the nauplius is ready to hatch, some eggs definitely not yet to the hatching point were tested by placing the egg cases in 0.5 *M* sucrose solution. The eggs of *Diaptomus ashlandi*, laid less than two hours previously by a gravid female, failed to shrink although they remained in the solution for an hour.

Two egg cases from *D. siciloides* were tested. Both contained eggs with embryos that were twitching and with eyes that were fully developed. In neither did shrinking take place at first. In one egg case there still was no shrinking after 17¼ hours, at which time it was replaced in lake water. No hatching took place, though the embryos were alive, as shown by the fact that they continued to twitch. After 8 hours the eggs were again placed in sucrose. Again no shrinkage occurred. When removed to lake water 16 hours later, the eggs appeared normal except that there was no twitching, but before long the embryos disintegrated.

In the second egg case containing twitching nauplii there was no shrinkage in 0.5 *M* sucrose at the end of an hour. In 2½ hours, however, 3 of the 16 eggs were shrunken. This egg case was thereupon return to lake water. By the time the solution was changed and the eggs located under the microscope, the shrunken eggs had swollen again, and one was beginning the process of hatching. Hatching then continued in egg after egg, and was perfectly normal in all instances except one, where the inner membrane after extrusion must have been perforated only slightly and lost its internal pressure slowly, collapsing completely around the larva, which struggled for a few seconds before it broke out.

These observations confirm that there is a change of permeability of the inner egg membrane at the time of hatching.

4. *Observations on the bursting of the inner egg membrane*

As discussed above, the nauplius always began its characteristic movements in normal hatching about a minute before the inner egg membrane burst and liberated it. It appeared as though the nauplius ruptured the membrane in some way by its activities, although it was not clear how this was done inasmuch as the rupture almost always occurred at the head end of the nauplius. Marshall and Orr (1954) said of this final act of hatching: "Quite suddenly it [nauplius] tears the membrane and swims away" (p. 393). Similarly, Ziegelmayer (1927) stated that the larva ruptured the inner membrane by the movements of its second antenna, but he thought the inner membrane was closely appressed around the nauplius after it was liberated from the outer membrane passively by an explosion-like bursting of the latter. In the observational section (section 1 above) of the present paper, the rupture of the membrane was implied to be the result of the struggling of the nauplius, because this was the way it appeared.

However, one of the experiments unexpectedly gave very revealing results. As reported in section 3 (above), an egg case in which the eggs were in the process of hatching was placed in 0.5 *M* sucrose for half an hour, then replaced in lake water. All the eggs but one hatched, but the nauplii were very weak. As many of the hatchings as possible were watched carefully and continuously until hatching was

completed. If the nauplii twitched or moved at all during hatching, they did so only by very slight and slow movements of the appendages. Three hatchings were followed where the entire process took place with no evidence of any muscular movement whatsoever on the part of the nauplius. Two other cases were similar, but there were some slight movements. These, however, were by no means sufficient to burst the membrane. The remaining hatchings could not be followed throughout (and one egg did not hatch). In spite of lack of naupliar movements, at the proper time the inner egg membrane burst and the nauplii were liberated.

A second egg case (*D. siciloides*) in which hatching was taking place was treated in the same manner. Of the 9 eggs in the egg sac, 8 shrank at once or very soon after immersion in the sucrose solution. One however, shrank only just before the case was returned to lake water, one-half hour later. In the lake water all of the 9 eggs hatched, although three of them had been "hatched" artificially by the inevitable rough treatment of rapidly changing solutions (these three, although appearing normal, never moved after liberation). Of the remaining six eggs, four hatched without any movements, and after hatching, three of these never moved (the fourth moved its appendages slightly during the process of dying, immediately after hatching). One of the nauplii twitched regularly, though weakly, before hatching, but during the period of the final bursting of the membrane there was no further movement, and the larva never moved after hatching. Only one of the nauplii (presumably from the egg that shrank at the last minute) hatched normally and lived indefinitely after hatching.

It is not believed that the bursting took place through the continued swelling of the sphere. Both before and after the above observations were made, numerous attempts were undertaken by measuring extruded spheres in normal eggs, to ascertain whether the swelling of the sphere continued until the time of breaking. No evidence of such growth after extrusion was obtained.

5. *Attempts to demonstrate the existence of a hatching enzyme*

In the observations and experiments described above, the hatching eggs were immersed in less than 0.5 cc. of lake water during hatching. There never was any evidence that the liberation of a hatching enzyme by the bursting of hatching eggs speeded up the hatching of those eggs in the cluster that still remained unhatched. In the eggs of *Diaptomus,* as reported above, the volume of the fluid within the inner egg membrane just before the nauplius was freed averaged 1,875,400 μ^3. This is less than 1/300,000 the volume of 0.5 cc. ($= 5 \times 10^{11}\ \mu^3$). With such a dilution of any hatching enzyme that might be present, one would hardly expect an effect.

Therefore, the volume of water involved was reduced (three experiments on *D. siciloides*) by drawing detached egg sacs in which the eggs were actively hatching into capillary tubes (i.d. = 1 mm.), along with half of an egg sac in which no hatching was occurring. The other half of the non-hatching batch of eggs was kept as a control.

In one of the three experiments the experimental eggs were in a rather early developmental stage. There were 10 experimental eggs and 15 hatching eggs enclosed in the capillary tube, with $12.7 \times 10^9\ \mu^3$ of water. Hence the ratio of fluid from the bursting membranes to the amount of diluting water was approximately 1:450. Neither the experimental eggs nor the controls hatched.

In the other two similar experiments the experimental eggs were in a very late stage of development. During the experimental period, hatching occurred in the experimental eggs some time after the other eggs had hatched. However, hatching took place almost simultaneously in the controls (in both instances hatching began first, and was completed first, in the experimental eggs, but the difference is not thought to be significant, inasmuch as some of the control eggs hatched before the last of the experimental ones). Thus, the existence of a hatching enzyme was not clearly demonstrated, in the conditions of these experiments.

Discussion

The results reported in the present paper fully confirm the osmotic nature of the hatching process in the eggs of copepods. The observations of Marshall and Orr (1954) on the events of hatching are supported and supplemented. No evidence was obtained in support of Ziegelmayer's (1927) contention that the outer membrane expanded osmotically while the inner membrane remained closely appressed around the enclosed nauplius. Furthermore, the observation reported by Ziegelmayer that the membrane began swelling 6 to 12 hours before hatching was not confirmed. Repeated attempts to detect an increase in volume of the egg previous to the few minutes before the hatching process was completed gave negative results. Marshall and Orr (1954) stated concerning the discrepancy between their observations and those of Ziegelmayer (p. 400): "It is difficult to decide whether Ziegelmayer was unable to see the bulging out of the inner membrane or whether the specimens he examined behaved in a different way." Ziegelmayer studied 17 (unlisted) species of *Cyclops*. Inasmuch as Marshall and Orr observed hatching in *Cyclops agilis* and *C. viridis*, and I observed it in *C. bicuspidatus* and *Mesocyclops edax*, and the behavior of all these was unlike that reported by Ziegelmayer, it would appear that his observations were faulty or deficient, either through the use of too little magnification, or through failure to follow through hatching in individual eggs.

On the other hand, the results reported above support Ziegelmayer's belief that hatching is initiated by a change of the permeability of the membrane. The lack of a similar conclusion by Marshall and Orr undoubtedly is associated with their lack of extensive experimentation.

From the above, two unsolved questions arise: 1) what is the origin and the nature of the dissolved material within the inner egg membrane that gives rise to a Δ_f of this fluid greater than the Δ_o of the external medium, and 2) what is the cause of the sudden change in the permeability of the inner membrane?

In fresh-water copepods, such as those reported here, the osmotic pressure of the fluid within the inner membrane conceivably could have its origin simply in the attainment of an equilibrium between Δ_f and Δ_i. However, the hatching of marine copepods, as reported by Marshall and Orr (1954), occurs in the same manner as that of the fresh-water forms. In most marine invertebrates the osmotic pressure of the internal medium is in equilibium with that of sea water, and there is no reason to believe that such species as *Calanus finmarchicus, Metridia longa,* and *Euchaeta norvegica,* which are stenohaline, are an exception. Therefore, in marine species, Δ_f must be greater than Δ_o, and greater than Δ_i at the time the egg was laid. No information exists at present bearing on the relation of Δ_i of the nauplius to Δ_f in these marine species just before hatching. Δ_i might either equal Δ_f, or it

might be less than Δ_f. A Δ_f that is greater than Δ_0 can be attained only by the action of the embryo or larva enclosed in the egg. It could result, as suggested by Marshall and Orr (1954), through the excretion of metabolic wastes by the embryo and/or nauplius, or there could be an active secretion of substances with osmotic value by special glands or gland cells (or both of these processes could be involved simultaneously). In view of the sudden change of permeability of the inner egg membrane described above, if excretory products are involved they need not be excreted suddenly as postulated by Marshall and Orr, but could accumulate gradually, and become osmotically effective suddenly through the rapid alteration of membrane permeability that initiates hatching. These matters can be settled only through further experimentation, particularly on stenohaline marine species of copepods.

It appears unlikely that a non-living membrane, such as the inner egg membrane of copepods, would be so constituted that its chemical or colloidal nature would suddenly be altered spontaneously at the proper time for hatching. If there is no spontaneous change, the influence for the alteration must come either from outside, or from the larva inside. Conceivably, the chemical nature of the membrane could be such that bacterial action from without would alter it in a definite course of time, but such an adaptation in evolution (especially considering that the bacterial population of waters is far from constant) seems far less likely than the evolution of a special hatching enzyme whose function is the chemical alteration of the membrane. Such a chemical alteration could change the membrane into a semi-permeable membrane (permeable to water) from its initial impermeable condition. It is true that the preliminary experiments described in section 5 above failed to detect the presence of such a hatching enzyme, but these experiments need repetition and refinement, and furthermore, from the nature of the experiment, negative results are not conclusive (although positive results would have been). The presence or absence of glands or gland cells producing a hatching enzyme has not been ascertained, but should be demonstrable histologically.

Hatching enzymes, such as are postulated above for the hatching of copepod eggs, have been proven to exist in certain fish eggs. Here, however, hatching apparently does not involve osmotic phenomena. The hatching enzyme, which is produced by special embryonic glands, digests the egg membranes, and the fry emerges more or less without the benefit of its own muscular movements (*e.g.*, see Bourdin, 1926 and Privolnev, 1943). Similar enzymes have been proven to exist in eggs of other aquatic animals, including *Rana pipiens* (Cooper, 1936). On the other hand, Wilson (1958) obtained results very similar to my own in the hatching of the eggs of the nematode, *Trichostrongylus retortaeformis*. Despite his negative results, he concluded from qualitative observations that some "hatching factor" is secreted which weakens the protein membrane before hatching. The hatching process itself in *T. retortaeformis* he thought to be osmotically determined.

Both Ziegelmayer and Marshall and Orr described the final rupture of the inner membrane as due to the active movements of the nauplius. The present results contradict this, and show that the hatching process can proceed to completion without any movements on the part of the enclosed nauplius. Although there apparently was no further increase of the volume of the fluid enclosed by the inner membrane during the final period of the hatching act, the possibility is not eliminated that there

was a continuation of the entry of osmotic water. With the membrane already stretched to its physical capacity, such a further entry would build up the internal pressure to the bursting point of the membrane. A further hypothesis suggests itself, however, namely that the membrane is destroyed chemically by a secretion from the anterior end of the larva. This would account for the fact the membrane almost invariably burst at the head end of the nauplius. These hypotheses also can be tested only by further experimentation.

Pyatakov (1926) studied hatching in the arguloid, *Argulus foliaceus*. Although his paper dealt primarily with the formation of the seam in one of the egg membranes along which splitting occurred during hatching, it is clearly implied that the hatching process itself is similar to that occurring in the Eucopepoda. Ziegelmayer (1927) reported, but did not describe, osmotic hatching in the eggs of an isopod (*Asellus*) and in an anostracan (*Branchipus*). Hall (1953) described hatching in the anostran *Chirocephalus,* and suggested that osmotic factors were involved. Przylecki (1921) and Ranult (1925) have presented results, summarized by Krogh (1939) and by Needham (1931), showing that in certain Cladocera, hatching is by osmotic means. In these forms, however, hatching differs considerably from that of the Copepoda, for it is the embryo itself that swells osmotically, and its increase in volume stretches the egg membrane until it bursts. A similar method of hatching was reported by Manton (1928) for *Hemimysis lamornae*. In some unpublished observations, the present author determined that hatching in the fresh-water decapod, *Palaemonetes kadiakensis*, occurs in part through osmosis. For a discussion of and references concerning osmotic hatching in other invertebrates see Needham (1931).

On the other hand, all Crustacea do not hatch osmotically. Le Roux (1933) described hatching in the amphipod *Gammarus,* where the young emerges from the egg by the active use of special egg teeth on the telson. This method was corroborated by the present author in the examination of hatching in *Gammarus fasciatus* in western Lake Erie.

Summary

1. The hatching process is described for the fresh-water copepods *Diaptomus ashlandi, D. siciloides, D. oregonensis, Cyclops bicuspidatus*, and *Mesocyclops edax*. In all of these species the inner membrane expands by the osmotic entry of water. The internal pressure thus produced ruptures the outer membrane, and the inner membrane containing the nauplius is extruded, forming a sphere whose volume is more than 2½ times that of the original egg. Subsequently the inner membrane bursts and the nauplius is thrown out.

2. It is shown that the osmotic pressure of the fluid within the expanded inner membrane is equivalent to that of a 0.03 to 0.04 *M* sucrose solution.

3. The inner membrane remains impermeable to water until the egg is ready to hatch. Thereupon the membrane changes its permeability within a short period of time. Hatching can be prevented indefinitely in eggs that are ready to hatch by immersing them in sufficiently concentrated sucrose solution.

4. Although during normal hatching the nauplius is active for a period of approximately a minute before hatching, this activity is not necessary for the completion of the hatching act. Nauplii hatched, even though they had been completely immobilized.

5. Attempts to demonstrate the presence of a hatching enzyme were unsuccessful.

6. It is suggested that the pre-hatching change in permeability of the membrane is caused by the action of chemicals produced by the larva. It is further suggested that the greater osmotic pressure of the fluid within the inner membrane is caused by external metabolites of the larva—either excretory or secretory.

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